Abscisic Acid Has a Key Role in Modulating Diverse Plant-Pathogen Interactions

Abscisic Acid Has a Key Role in Modulating Diverse Plant-Pathogen Interactions[1][C][W][OA]

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We isolated an activation-tagged Arabidopsis (*Arabidopsis thaliana*) line, *constitutive disease susceptibility2-1D* (*cds2-1D*), that showed enhanced bacterial growth when challenged with various *Pseudomonas syringae* strains. Systemic acquired resistance and systemic *PATHOGENESIS-RELATED GENE1* induction were also compromised in *cds2-1D*. The T-DNA insertion adjacent to *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE5* (*NCED5*), one of six genes encoding the abscisic acid (ABA) biosynthetic enzyme NCED, caused a massive increase in transcript level and enhanced ABA levels >2-fold. Overexpression of *NCED* genes recreated the enhanced disease susceptibility phenotype. *NCED2*, *NCED3*, and *NCED5* were induced, and ABA accumulated strongly following compatible *P. syringae* infection. The ABA biosynthetic mutant *aba3-1* showed reduced susceptibility to virulent *P. syringae*, and ABA, whether through exogenous application or endogenous accumulation in response to mild water stress, resulted in increased bacterial growth following challenge with virulent *P. syringae*, indicating that ABA suppresses resistance to *P. syringae*. Likewise ABA accumulation also compromised resistance to the biotrophic oomycete *Hyaloperonospora arabidopsis*, whereas resistance to the fungus *Alternaria brassicicola* was enhanced in *cds2-1D* plants and compromised in *aba3-1* plants, indicating that ABA promotes resistance to this necrotroph. Comparison of the accumulation of salicylic acid and jasmonic acid in the wild type, *cds2-1D*, and *aba3-1* plants challenged with *P. syringae* showed that ABA promotes jasmonic acid accumulation and exhibits a complex antagonistic relationship with salicylic acid. Our findings provide genetic evidence that the abiotic stress signal ABA also has profound roles in modulating diverse plant-pathogen interactions mediated at least in part by cross talk with the jasmonic acid and salicylic acid biotic stress signal pathways.

Plants rely on efficient resistance mechanisms that involve multiple layers of constitutive and induced defenses to protect themselves from pathogen attacks. Constitutive physical and chemical barriers on the plant surface prevent the establishment of pathogen infection structures, whereas the effect of induced defense is based on the ability to perceive and respond to pathogen-derived factors. The basal perception systems at the plant cell surface recognize general microbial invaders by detecting conserved microbe-associated molecular patterns (MAMPs), such as flagellin, a structural component of the bacterial flagellum (Gómez-Gómez and Boller, 2002). A multitude of plant major disease resistance (R) proteins specify recognition of pathogens carrying the corresponding avirulence (*avr*) genes. Recognition of the MAMPs- and *avr*-dependent signals may lead to activation of some overlapping inducible defenses, including synthesis or mobilization of antibiotic compounds and deposition of callose to reinforce the cell wall (Nurnberger et al., 2004; Clay et al., 2009). The MAMP-triggered basal defenses, however, do not always result in the development of localized programmed cell death (referred to as the hypersensitive response [HR]), a characteristic feature generally associated with *R*-gene-dependent defenses. HR also contributes to the establishment of the long-lasting systemic acquired resistance against subsequent attack by a broad range of normally virulent pathogens (Durrant and Dong, 2004).

Complex signaling networks orchestrate different types of plant-inducible defenses to prevent microbial growth. Pathogen recognition triggers a number of rapid cellular responses, including ionic changes, and phosphorylation cascades, which precede the accumulation of reactive oxygen species, nitric oxide, and salicylic acid (SA) and the transcriptional activation of defense-related genes. Interplay between reactive oxygen species, nitric oxide, and SA contributes to the establishment of HR. SA also has a key role in establishing local and systemic resistance to many

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virulent biotrophic pathogens, whereas jasmonic acid (JA) and ethylene (ET) are more often associated with resistance to necrotrophic pathogens. Considerable interactions occur within and between these hormone signaling networks, resulting in an overall mutual antagonism between SA and JA/ET signaling (Kunkel and Brooks, 2002; Beckers and Spoel, 2006). The similarities between the mechanisms of signaling and defense that underlie multilayer of disease resistance and the close interactions between different signaling pathways imply a fine-tuned deployment of conserved defense signals and effectors in different plant-pathogen interactions.

Genetic dissection of disease resistance in the model plant Arabidopsis (*Arabidopsis thaliana*) through loss-of-function mutagenesis has identified some important components of basal and *R*-gene-dependent defenses. For example, *EDS1*, *PAD4*, and *MOS3* are essential for the resistance specified by the subclass of nucleotide-binding site Leu-rich repeat R proteins that contain an N-terminal Toll Interleukin1 receptor domain, and they are also required for basal resistance to virulent pathogens (Dangl and Jones, 2001; Glazebrook, 2001; Zhang and Li, 2005), while *NPR1* was shown to mediate SA-dependent defense responses (Dong, 2001). However, many genes are hard to identify by this approach due to lethality or functional redundancy, especially in complex and reiterative signal networks (Dangl and Jones, 2001).

T-DNA activation tagging generates dominant, gain-of-function mutations that lead to enhanced expression of the tagged genes (Weigel et al., 2000). This approach has been used to investigate resistance mechanisms with the expectation that elevated expression of defense signaling molecules may lead to a quantitative impact on resistance even where there is significant cross talk and functional redundancy. Thus, several new components have been shown to regulate plant-pathogen interactions (Grant et al., 2003; Xia et al., 2004; Zhang et al., 2007). Aiming to uncover genes involved in SA-mediated signaling mechanisms, we carried out activation tagging in SA-deficient Arabidopsis plants that constitutively express the bacterial salicylate hydroxylase (NahG; Gaffney et al., 1993). No mutants were recovered that had enhanced resistance to infection by virulent bacteria; instead, we identified a mutant with enhanced disease symptoms that supported increased bacterial growth compared to *NahG* plants. Molecular characterization of the mutant revealed that massive activation of *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE5* (*NCED5*) led to constitutive accumulation of high levels of endogenous abscisic acid (ABA), which impacted multiple layers of defense against diverse plant pathogens. Further investigation showed that endogenous ABA synergizes with JA and exhibits a complex antagonistic relationship with SA during disease development. Our findings provide genetic evidence that physiological levels of ABA play an important role in modulating diverse plant-pathogen interactions and elaborate a link between abiotic stress and level of disease susceptibility.

RESULTS

cds2-1D Is a Dominant Mutation Conferring Enhanced Disease Susceptibility to *Pseudomonas syringae*

A collection of 8,000 activation tagged T1 plants in the *NahG* background were screened by hand-infiltration with virulent *P. syringae* pv *maculicola* ES4326 (*Psm*) chromosomally tagged with *luxCDABE* (Fan et al., 2008). While no mutant was found that enhanced basal resistance in the absence of SA, a mutant with increased disease severity was identified. In the F2 generation following back crossing to Columbia-0 (Col-0), three-quarters of the 40 plants in which *NahG* had been segregated out showed severe tissue maceration when challenged with *Psm*, while one-quarter developed only mild chlorosis akin to that in wild-type plants (Fig. 1A). Basta resistance, which is conferred by the selectable marker of the activation tagging vector, cosegregated with enhanced symptom development. Thus, the disease phenotype reflects a dominant mutation associated with the single T-DNA insertion and independent of the *NahG* transgene. We designated this mutant *constitutive disease susceptibility2-1D* (*cds2-1D*).

Disease Phenotypes of *cds2-1D*

Quantitative analysis of bacterial growth showed >30-fold greater growth of both virulent *Psm* and virulent *P. syringae* pv *tomato* DC3000 (*Pst*) in hand-infiltrated leaves of *cds2-1D* compared to wild-type plants, accompanied by severe tissue maceration. The growth of *Psm* carrying the *avrRpm1* avirulence gene (*Psm-avrRpm1*), which triggers HR on the Col-0 genotype due to the cognate *Rpm1* disease resistance gene, was 6-fold greater in *cds2-1D* than in wild-type plants, indicating a weakened resistance to a normally avirulent bacterial strain. The type three secretion system (TTSS)-deficient *hrcC* mutant of *Pst* DC3000 (*hrcC*) grew to 20-fold higher levels on *cds2-1D* plants compared to Col-0 plants, but the growth of this nonpathogenic mutant was weak compared to wild-type *Pst* and did not result in tissue maceration or development of visible lesions. Likewise, growth of *P. syringae* pv *glycinea* (*Psg*), which is nonpathogenic on Arabidopsis, was only enhanced 3-fold in *cds2-1D* compared to very low levels of growth in wild-type plants (Fig. 1B).

Accumulation of *PATHOGENESIS-RELATED GENE1* (*PR1*) transcripts is a marker for SA signaling and establishment of systemic acquired resistance. Induction of *PR1* by spraying plants with 1 mM SA was strongly suppressed in *cds2-1D* plants compared to wild-type Col-0 (Fig. 2A). Furthermore, while the local induction of *PR1* at the site of inoculation with *Psm-avrRpm1* was not affected in *cds2-1D*, the mutant plants

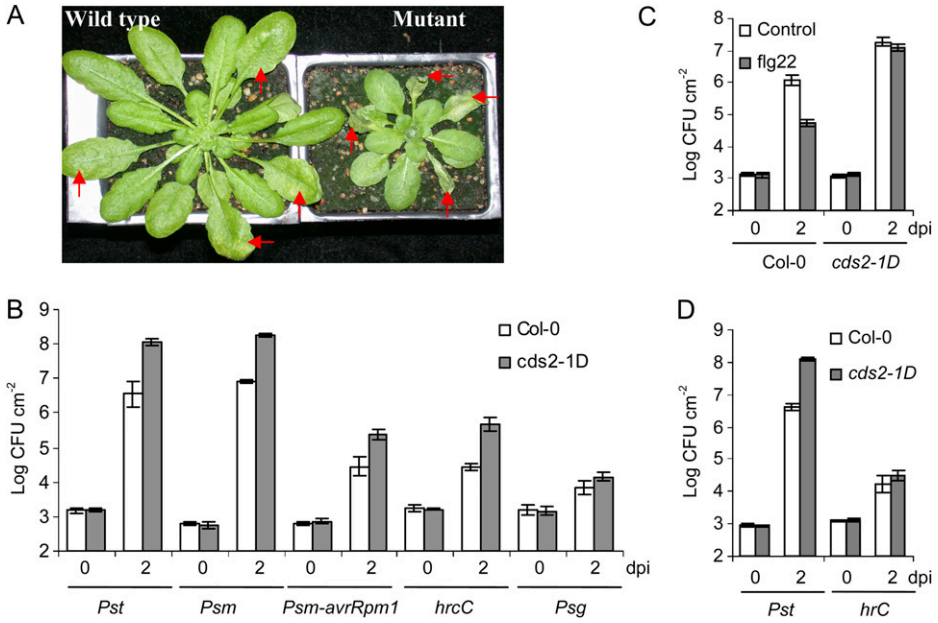


Figure 1. Compromised disease resistance to bacterial infection in *cds2-1D* mutant. A, Disease symptom of wild-type Col-0 (left) and mutant *cds2-1D* (right) plants 3 d after hand-infiltration of *Pst*. The arrows indicate the inoculated leaves. B, Bacterial growth of various *P. syringae* strains in leaves from *cds2-1D* mutant (gray) and wild-type Col-0 (white) plants. C, Flagellin-induced restriction of *Pst* growth is attenuated in *cds2-1D* mutant. Leaves of Col-0 and *cds2-1D* plants were pretreated for 24 h by infiltration with 100 nM flg22 peptide (gray) or water as control (white) before bacterial challenge. D, Bacterial growth of wild-type *Pst* and the TTSS-deficient *hrcC* strain in detached leaves from Col-0 (white) and *cds2-1D* (gray) plants. The hand-infiltrated leaves were excised from plants and inserted in 0.7% water-agarose plate after the excessive water had dissipated. The plates were sealed with 3M Micropore surgical tape and incubated under the same condition as the in planta bacterial growth assay. In all the above experiments, leaves were infiltrated with 10^5 cfu mL^{-1} of bacteria and were collected 2 d after inoculation for growth assay. Data shown are means ± SD, and similar results were observed in three replicate experiments. dpi, Days post inoculation.

showed no systemic accumulation of *PR1* transcripts (Fig. 2B). Likewise, following local induction with avirulent *Psm-avrRpm1*, systemic acquired resistance of distant leaves subsequently challenged with virulent *Pst* was substantially reduced in *cds2-1D* plants compared to wild-type plants (Fig. 2C).

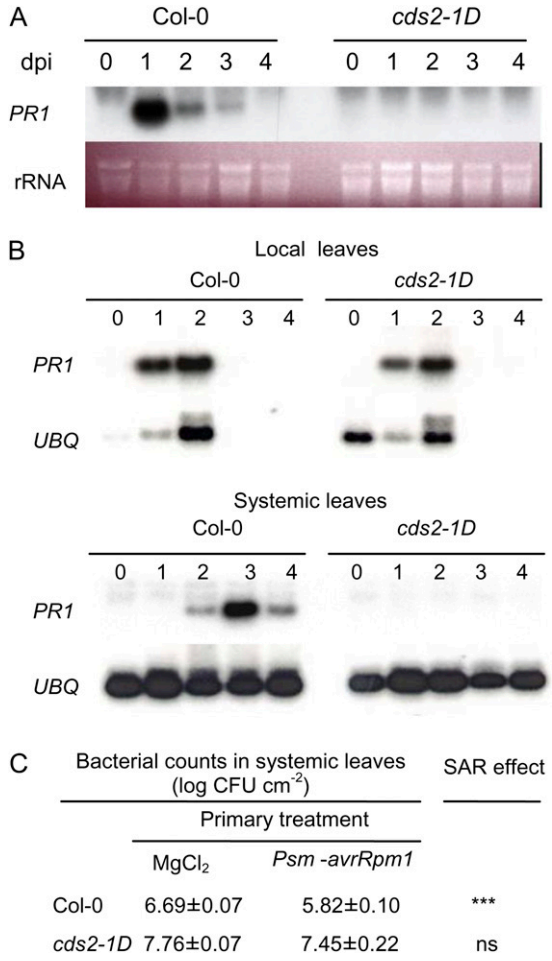
The observation of enhanced growth of both virulent and *hrcC* strains on *cds2-1D* plants prompted us to check whether the mutation also affects MAMP-induced basal resistance, which requires SA signaling to suppress *hrcC* growth but is largely independent of SA pathways in suppressing growth of virulent bacteria (Zipfel et al., 2004; Tsuda et al., 2008). Infiltration of leaves with 100 nM flg22 peptide 1 d before bacterial challenge reduced the growth of virulent *Pst* >10-fold in wild-type Col-0 plants, while the same treatment had little or no effect on *cds2-1D* mutant plants (Fig. 1C), indicating compromised MAMP-triggered immunity.

Other *cds2-1D* Phenotypes

Apart from being more susceptible to *Pst* and *Psm*, *cds2-1D* plants showed delayed development and were smaller than the wild type. Moreover, when *cds2-1D* plants were well watered and kept in darkness, leaves became water soaked. To examine if this phenotype was associated with enhanced disease susceptibility, we tested bacterial growth in excised leaves, which do not develop water-soaked leaves. Wild-type *Pst* grew to 30-fold higher levels on detached *cds2-1D* leaves than equivalent wild-type leaves, but there was no significant difference between the weak growth of the *hrcC* mutant on detached leaves from *cds2-1D* and wild-type plants (Fig. 1D). Thus, most of the enhanced growth of *Pst* on *cds2-1D* was independent of the water-soaking phenotype but required a functional TTSS to be realized, with a smaller TTSS-independent component associated with the water-soaking phenotype (Fig. 1, B and D).

Molecular Characterization of the *cds2-1D* Mutation

The *cds2-1D* phenotype and Basta resistance cosegregated as simple dominant Mendelian traits, suggesting that the *cds2-1D* locus was T-DNA tagged. Thermal asymmetric interlaced PCR (Liu et al., 1995) and inverse PCR (IPCR) failed to amplify Arabidopsis DNA sequences but revealed the presence of left and right T-DNA borders, indicating rearrangement at the



C

	Bacterial counts in systemic leaves (log CFU cm^{-2})		SAR effect
	Primary treatment		
	$MgCl_2$	*Psm -avrRpm1*	
Col-0	6.69±0.07	5.82±0.10	***
cds2-1D	7.76±0.07	7.45±0.22	ns

Figure 2. SA induced *PR1* expression and systemic acquired resistance (SAR) is suppressed in the *cds2-1D* mutant. Leaves were collected at 0, 1, 2, 3, and 4 d after SA treatment or bacterial challenge. A, RNA gel blot analysis of *PR1* gene transcript levels in wild-type Col-0 and *cds2-1D* mutant plants sprayed with 1 mM SA. dpi, Days post inoculation. B, Systemic induction of *PR1* expression is suppressed in the *cds2-1D* mutant plant. Two leaves of each plant were inoculated with 5×10^6 cfu mL^{-1} of *Psm-avrRpm1*. The inoculated local leaves and noninoculated systemic leaves were collected for RNA gel blot assay of *PR1* transcript levels. C, Attenuated systemic acquired resistance in *cds2-1D* mutant. Treatments consist of inoculation of two leaves with 5×10^6 cfu mL^{-1} of avirulent *Psm-avrRpm1*, followed 2 d later with the second inoculation of systemic leaves with 10^5 cfu mL^{-1} of virulent *Pst*. Magnesium chloride (10 mM) was infiltrated as control to avirulent bacterial treatment. Growth of virulent *Pst* in systemic leaves was monitored 2 d after the second inoculation. Significant reduction of *Pst* growth in systemic leaves was observed in Col-0 plants between $MgCl_2$ and avirulent *Psm-avrRpm1* treatments (indicated by ***, *t* test, $n = 6$, $P < 0.001$), whereas the difference in *cds2-1D* plants was not statistically significant (indicated by "ns"). This experiment was repeated twice with similar results. [See online article for color version of this figure.]

insertion site. DNA gel blots hybridized with *Cauliflower mosaic virus* (*CaMV*) 35S enhancer sequences positioned the T-DNA region adjacent to the putative rearranged insertion site, and using IPCR with primers anchored in the identified T-DNA region, we were able to isolate Arabidopsis sequences flanking the insertion site. As illustrated in Figure 3A, the T-DNA had inserted approximately 400 bp upstream of the gene corresponding to locus *At1g30100* such that the *CaMV* 35S enhancers were approximately 3.5 kb from the tagged gene. No other gene within 9 kb either side of this locus was annotated in The Arabidopsis Information Resource database. *At1g30100* sequences hybridized to an approximately 2-kb transcript in RNA blots of total RNA from healthy leaves of *cds2-1D* plants, whereas little or no hybridization was observed with an equivalent RNA sample from wild-type plants (Fig. 3B).

At1g30100 Is a Pathogen-Induced Member of the *NCED* Family

The *At1g30100* locus is *NCED5*, one of six Arabidopsis genes encoding NCED. NCED catalyzes oxidative cleavage of 9-cis-xanthophylls to form xanthoxin, a key regulatory step of ABA biosynthesis. Among these six homologs, *NCED3* is a major drought-induced *NCED* in Arabidopsis leaves, and *NCED* genes are developmentally regulated and associated with ABA synthesis in roots, flowers, and developing seeds (Iuchi et al., 2001; Tan et al., 2003). Based on sequence similarities *NCED5* and *NCED3* are members of a distinct subfamily of four of the *NCED* genes, and the transcription profiles of this subfamily in response to bacterial challenge were investigated by reverse transcription (RT)-PCR. As shown in Figure 3C, *NCED5* and *NCED2* transcripts accumulated in the later stages of the compatible interaction with *Psm* but were not induced by avirulent *Psm-avrRpm1*. In contrast, *NCED3* showed strong early expression in both compatible and incompatible interactions, while *NCED9* transcripts did not accumulate in response to bacterial inoculation.

Overexpression of *NCED5* and *NCED3* Enhances Susceptibility to *P. syringae*

To confirm that *NCED* overexpression indeed enhances disease susceptibility, the coding sequences of *NCED5*, the T-DNA-tagged gene, and *NCED3*, which is strongly induced *by P. sryingae*, were expressed in transgenic plants under the control of the *CaMV* 35S promoter. Multiple independent transgenic lines showing *cds2-1D*-like phenotypes were identified for both constructs, and growth of *Psm* was enhanced >10-fold in plants, showing strong constitutive expression of either *NCED3* or *NCED5* compared to growth on equivalent wild-type control plants (Fig. 3D).

To investigate whether enhanced disease susceptibility could be separated from the developmental phenotypes also associated with strong constitutive *NCED* overexpression, we made transgenic plants with *NCED3* under the control of the estradiol-inducible XVE system (Zuo et al., 2000). No difference was observed

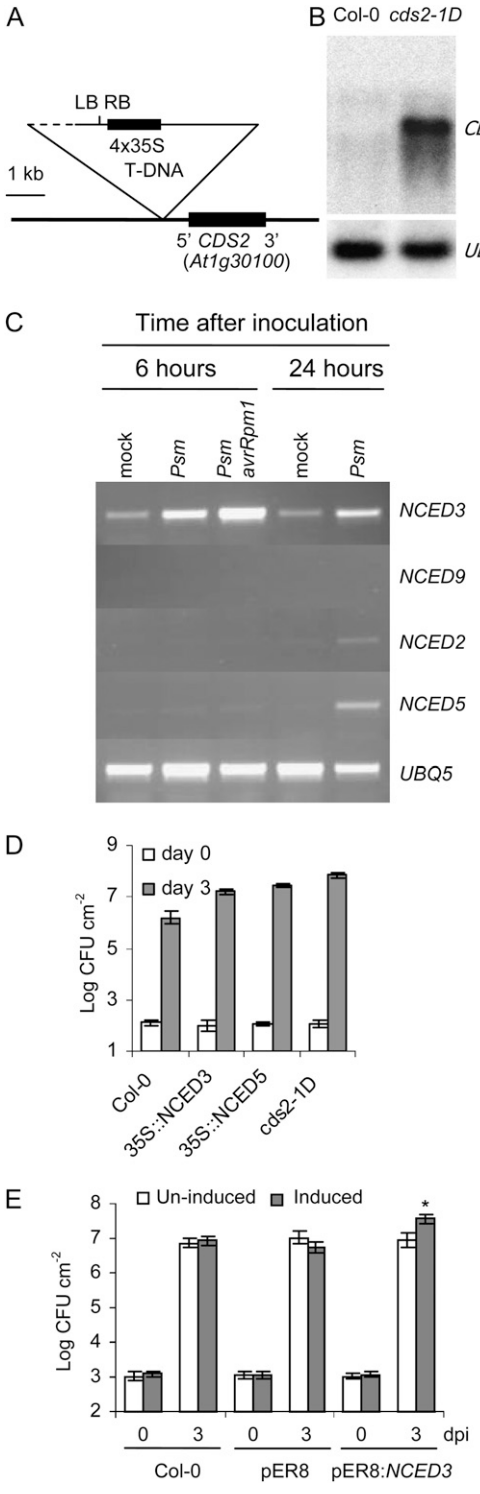


Figure 3. Functional analysis of *cds2-1D* allele and the related gene family members involved in Arabidopsis-*P. syringae* interaction. A, Structure of T-DNA insertion in *cds2-1D* mutant. The detected head-to-head T-DNA left and right borders are marked as LB and RB, respectively. The uncharacterized T-DNA region is indicated by dashed line. B, *CDS2* expression is massively enhanced in the *cds2-1D* mutant. RNA gel blot assay of *CDS2* gene transcript in healthy leaves from Col-0 and *cds2-1D* mutant plants. The bottom panel shows ubiquitin transcript levels of the same samples. C, Arabidopsis *NCED* genes are induced by bacterial infection. RT-PCR analysis of *NCED* transcripts levels in leaves from Col-0 plants that were infiltrated with 5×10^7 cfu mL^{-1} of virulent *Psm* or avirulent *Psm-avrRpm1*. Water was infiltrated as mock treatment. No PCR product could be observed when non-reverse-transcribed total RNA was used as negative controls to amplify *NCED* genes (data not shown). D, Constitutive overexpression of *NCED* genes enhanced susceptibility to bacterial infection. One of each representative transgenic lines constitutively overexpressing *NCED3* or *NCED5* genes, together with wild-type Col-0 and *cds2-1D* mutant plants, was hand-infiltrated with 10^4 cfu mL^{-1} of virulent *Psm*, and in planta bacterial growth were determined at 3 d after inoculation. E, Conditional overexpression of *NCED3* enhances susceptibility to bacterial infection. Wild-type Col-0 plants and plants transformed with empty vector pER8 or pER8-*NCED3* were sprayed with 10 μM β-estrodial 1 d before hand-infiltration of virulent *Pst* at 10^5 cfu mL^{-1}. Water was sprayed as uninduced control. The in planta bacterial growth was determined 3 d after inoculation. Significant differences in bacterial growth were only detected between control and induced pER8:NCED3 plants (indicated by *, *t* test, $P < 0.05$). dpi, Days post inoculation. Data shown in the bacterial growth assays are means $\pm$ SD, and each experiment was repeated at least twice with similar results.

in the development or morphology of pER8-*NCED3* transgenic plants compared to the wild type. Spraying pER8-*NCED3* lines with 10 μM of β-estradiol induced strong accumulation of *NCED3* transcripts (data not shown), and challenge with *Pst* 24 h after estradiol treatment resulted in 4-fold greater bacterial growth than in equivalent uninduced plants. Estradiol treatment of wild-type or empty vector control transgenic plants had no effect on susceptibility to *Pst* (Fig. 3E).

Physiological Role of ABA in Bacterial Susceptibility

Overexpression of *NCED* enhances ABA levels (Thompson et al., 2000; Iuchi et al., 2001); likewise, leaves of healthy *cds2-1D* plants showed 3-fold elevation in the level of ABA (Fig. 4A). To further investigate if the enhanced susceptibility to bacterial infection in *cds2-1D* plants was tightly associated with the elevated level of endogenous ABA, we crossed *cds2-1D* with *aba1-1* and *aba2-1*, which carry genetic lesions in the ABA biosynthetic pathway (Léon-Kloosterziel et al., 1996; Marin et al., 1996). All of the F1 plants derived from the crosses were Basta resistant and showed *cds2-1D* morphology, while in F2 progenies, *aba* genotyping analysis of the Basta-resistant plants showed that homozygosity of either *aba* locus resulted in the morphological phenotypes characteristic of the *aba* mutant. Similarly, both *aba1-1* and *aba2-1* mutations eliminated enhanced bacterial disease susceptibility of *cds2-1D* (Fig. 4B), indicating that the observed *cds2-1D* phenotypes are dependent on ABA biosynthesis. These findings, together with the observation that some *NCED* genes are pathogen induced, prompted further investigation of the role of ABA in bacterial pathogenesis.

First, we checked whether exogenous ABA promoted bacterial growth. To introduce ABA, leaves of wild-type Col-0 plants were detached after hand-

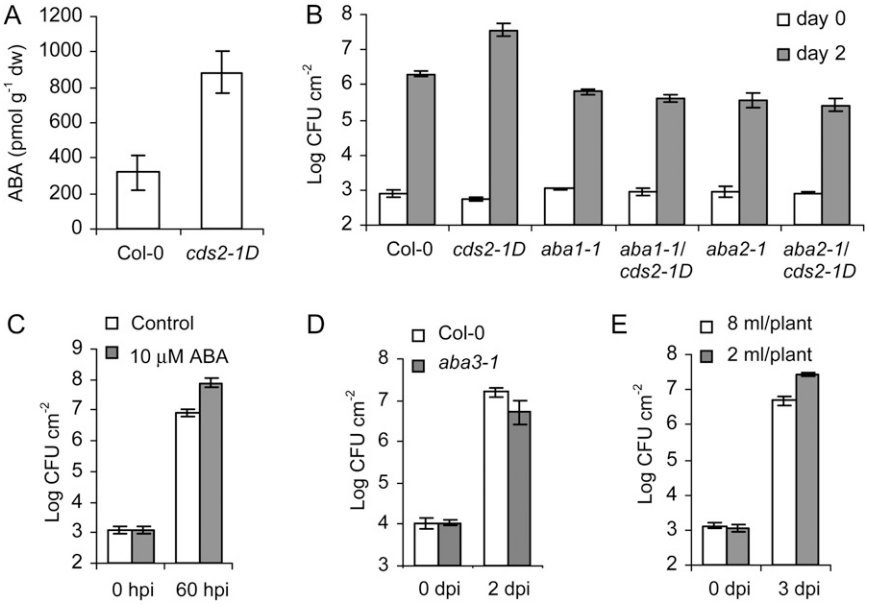


Figure 4. Physiological role of ABA in bacterial susceptibility. A, The *cds2-1D* mutant plants have increased basal level of free ABA in comparison to wild-type Col-0 plants. B, The enhanced bacterial growth in *cds2-1D* mutant is dependent on ABA biosynthesis pathway. Leaves of wild-type and homozygous mutant plants were hand-infiltrated with 10^5 cfu mL^{-1} of virulent *Pst*, and bacterial growth was determined 2 d after inoculation. C, Exogenous ABA treatment enhances bacterial susceptibility. Leaves of wild-type Col-0 plants were hand-infiltrated with 10^5 cfu mL^{-1} of virulent *Pst* before they were excised from the plants with a razor blade and fed through the petiole with sterilized water (white) or 10 μM ABA (gray). Bacterial number in the inoculated leaves was determined 60 h after inoculation. D, Bacterial susceptibility is attenuated in ABA-deficient mutant plants. Leaves from wild-type Col-0 (white) and *aba3-1* mutant (gray) plants were hand-infiltrated with 10^6 cfu mL^{-1} of virulent *Pst*, and the leaf bacterial number was determined 2 d after inoculation. E, Water restriction enhanced bacterial susceptibility in Arabidopsis. Five-week-old Col-0 plants were supplied daily with sufficient level (8 mL/plant, white) or restricted level (2 mL/plant, gray) of water for 1 week and inoculated with 10^5 cfu mL^{-1} of virulent *Pst*. Bacterial number in the inoculated leaves was determined 3 d after inoculation. Data shown in the above assays are means ± SD; g^{-1}dw is per gram dry weight. All the experiments were repeated at least twice with similar results. dpi, Days post inoculation; hpi, hours post inoculation.

infiltration with virulent *Pst* and petioles immersed in 10 μM ABA or water. After 2 d, bacterial growth was 10-fold greater in ABA-treated leaves compared to water-treated leaves (Fig. 4C). Similar effects could be observed in ABA-treated leaves on growth of the *hrcC* strain (Supplemental Fig. S1A).

Next, we used liquid chromatography-mass spectrometry (LC-MS) to determine whether ABA accumulated during disease development. Marked accumulation of ABA was observed starting 12 h after inoculation with virulent *Pst* with continuing accumulation as chlorotic lesions developed. ABA accumulation preceded the onset of JA accumulation during lesion development but was slower than the transient accumulation of SA in the early stages of the compatible interaction. No significant changes in ABA, SA, or JA levels were observed in equivalent mock-inoculated plants (Fig. 5A).

Third, we investigated whether susceptibility to *P. syringae* was affected when ABA synthesis was blocked. The Arabidopsis mutant *aba3-1* is blocked in the last step of ABA biosynthesis and fails to accumulate ABA in response to dehydration (Xiong et al., 2001). Three days after challenge with *Pst*, leaves of *aba3-1* plants exhibited only weak symptom development compared to the chlorosis observed in equivalent leaves of wild-type controls (Supplemental Fig. S1B). Reduced symptom development was accompanied by 3-fold less bacterial growth in *aba3-1* plants compared to the wild type (Fig. 4D).

Finally, we investigated whether mild water stress, a physiological condition that promotes accumulation of ABA, affected disease susceptibility. Wild-type plants were stressed by a reduced watering regimen for 7 d prior to challenge with *Pst*. Three days after inoculation bacterial growth was 5-fold greater than in unstressed plants (Fig. 4E).

Effect of ABA in Other Pathosystems

Taken in aggregate, these findings indicated that ABA enhanced susceptibility to the hemibiotrophic bacterial pathogen *P. syringae*. To extend the analysis, we investigated whether ABA also affected susceptibility in other pathosystems. Pathogenesis of the oomycete biotroph *Hyaloperonospora arabidopsis* was

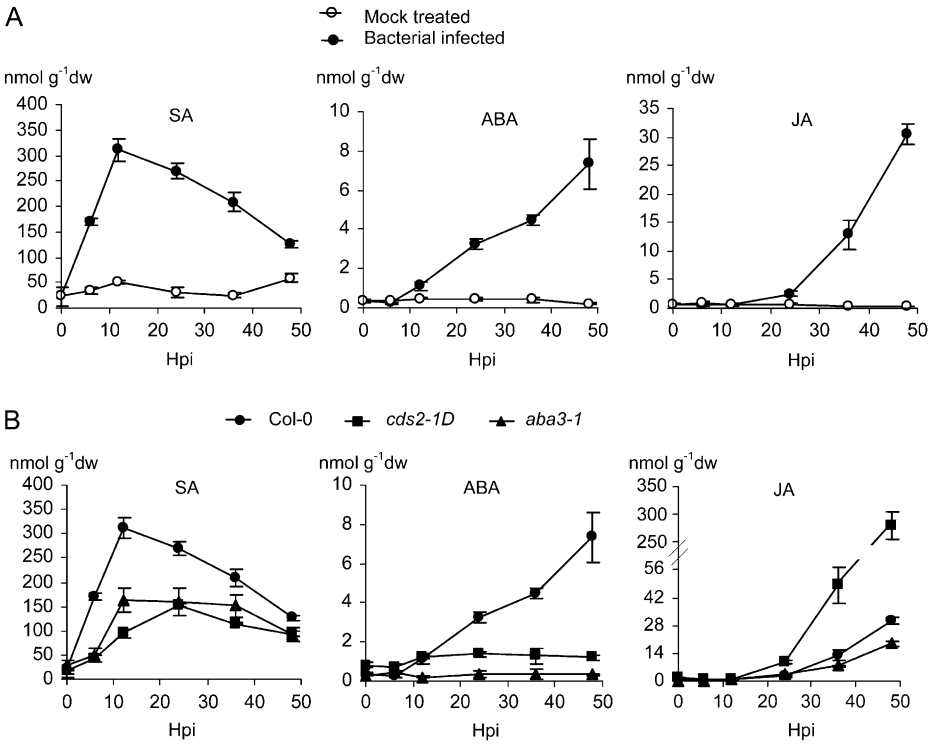


Figure 5. Accumulation of free SA, ABA, and JA in Arabidopsis leaves infected with *P. syringae*. Leaves of 5-week-old plants were hand-infiltrated with water or 5×10^6 cfu mL^{-1} of virulent *Pst*. The inoculated leaves were collected at 0, 6, 12, 24, 36, and 48 h post inoculation (Hpi) and the extracts of leaf tissue used for LC-MS quantification of free SA, ABA, and JA. A, SA, ABA, and JA induction by bacterial infection (solid circle) of wild-type Col-0 plants. Water was infiltrated as mock treatment (open circle). B, Comparison of SA, ABA, and JA induction in wild-type Col-0, *aba3-1* (solid triangle), and *cds2-1D* (solid square) mutant plants in response to bacterial infection. For each data point, three biological replicates were assayed, and data shown are means ± SD; g^{-1}dw is per gram dry weight. The experiments were repeated twice with similar outcomes.

suppressed in leaves of *aba3-1* plants with 3-fold less sporulation than in wild-type plants (Fig. 6A). While *H. arabidopsis* sporulation was not significantly enhanced in leaves of *cds2-1D* plants, hyphal growth was substantially more prolific in leaves of *cds2-1D* compared to wild-type plants (Fig. 6A; Supplemental Fig. S1C). In contrast, symptom development following inoculation with the fungal necrotroph *Alternaria brassicicola* was enhanced in *aba3-1* plants and suppressed in *cds2-1D* plants compared to wild-type controls (Fig. 6B).

Endogenous ABA Synergizes with JA and Exhibits a Complex Antagonistic Relationship with SA during Disease Development

The observation that ABA reduced susceptibility to the necrotrophic pathogen *A. brassicicola* while promoting susceptibility to the biotrophic pathogens *P. syringae* and *H. arabidopsis* is reminiscent of some features of JA function in plant-pathogen interactions and the often antagonistic effects between JA and SA. We therefore examined the interplay between ABA and these two biotic stress signal molecules. In *Pst*-inoculated wild-type Col-0 plants following transient SA induction, ABA accumulation preceded the onset of JA induction (Fig. 5A), suggesting that ABA may promote JA accumulation and enhance JA action. To test this hypothesis, we examined JA accumulation in genotypes with differing ABA levels. *Pst* induction of JA accumulation was reduced in ABA-deficient *aba3-1* plants compared to the wild type, whereas in *cds2-1D* plants, *Pst* induction of JA accumulation was accelerated and markedly potentiated (Fig. 5B). As expected, there was little accumulation of ABA in *aba3-1* plants challenged with *Pst*, but interestingly, infection of *cds2-1D* plants did not enhance ABA levels beyond the elevated basal level in healthy leaves, suggesting that the levels of ABA in the early stages of infection are critical to the outcome and possible operation of a feedback loop (Fig. 5B). Building on this, and in contrast to the simple relationship between ABA and JA, there appeared to be a complex relationship between SA and ABA. Thus, while early SA accumulation was weaker in *cds2-1D* plants than the wild type, ABA-deficient plants also showed reduced SA accumulation in response to *Pst* challenge (Fig. 5B).

DISCUSSION

ABA is an important phytohormone that regulates many aspects of plant growth and development, especially responses to various abiotic stresses. Recently, several studies have demonstrated that ABA may also be widely involved in plant responses to biotic stresses caused by a broad range of plant pathogens (Mauch-Mani and Mauch, 2005; Asselbergh et al., 2008a) or insect herbivores (Thaler and Bostock, 2004; Bodenhausen and Reymond, 2007). Many early studies relied on the application of exogenous ABA to increase plant ABA levels (Asselbergh et al., 2008a), although exogenous ABA may not exert the same physiological effects as

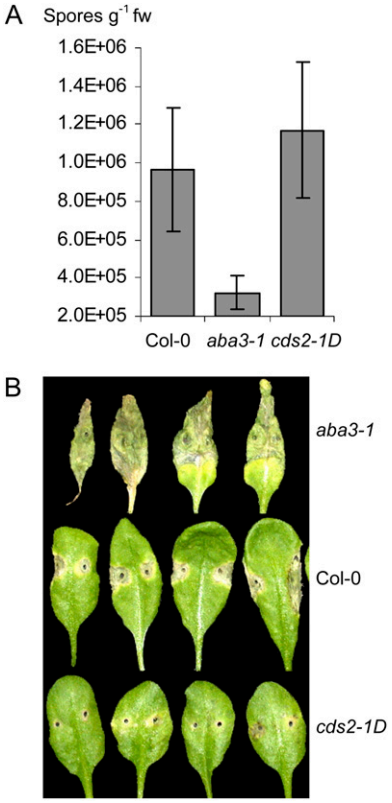


Figure 6. ABA is involved in other Arabidopsis-microbial interactions. A, ABA biosynthesis is required for *H. arabidopsis* to attain full virulence on Arabidopsis. Three-week-old wild-type Col-0, *aba3-1*, and *cds2-1D* seedlings were sprayed with 4×10^4 spores mL^{-1} of *H. arabidopsis*, and the leaves were collected at 7 d after inoculation to determine levels of pathogen sporulation. Data shown are means ± SD; g^{-1}fw is per gram fresh weight. B, ABA is required for resistance to *A. brassicicola* in Arabidopsis. Leaves of 6-week-old plants were inoculated with 5 μL of 10^5 spores mL^{-1} of *A. brassicicola*, and 7 d after the inoculated leaves from one representative plant of *aba3-1* (top), Col-0 (middle), and *cds2-1D* (bottom) genotypes were excised for photography. These experiments were repeated twice with similar outcomes.

endogenous ABA (Christmann et al., 2005), and this may potentially complicate the interpretations of the roles of ABA in plant-pathogen interactions (Wasilewska et al., 2008). Our work on the *cds2-1D* mutant, which was identified from a forward genetic screen, showed that activation of NCED5, a key enzyme regulating de novo ABA biosynthesis, led to elevated endogenous ABA levels and to enhanced susceptibility to infection by *P. syringae*. The observations that overexpression of NCEDs in transgenic Arabidopsis recapitulated *cds2-1D* disease phenotype and that genetic lesions in the ABA biosynthesis pathway abolished the enhanced bacterial growth in the *cds2-1D* mutant pinpoint the role of ABA in promoting susceptibility to this hemibiotrophic bacterial pathogen. Susceptibility was increased by application of exogenous ABA at physiological levels or by endogenous accumulation in response to mild water stress, thereby demonstrating the physiological relevance of ABA-mediated suppression of resistance to this hemibiotrophic bacterial pathogen. Thus, our data help establish a potentially important link between abiotic stress and the level of host susceptibility to disease.

Compared with abiotic stresses, however, the role of ABA in biotic stresses may vary among different pathosystems. In mutants deficient in ABA biosynthesis, the resistance to the biotrophic pathogens *Hyaloperonospora parasitica* and *Blumeria graminis* is enhanced (Mohr and Cahill, 2003; Jensen et al., 2008), while resistance to the necrotrophic pathogens *A. brassicicola* and *Pythium irregulare* is compromised (Ton and Mauch-Mani, 2004; Adie, et al., 2007). Our data also demonstrate that ABA modulates pathogenesis in diverse plant-pathogen interactions with outcomes apparently influenced by the infection biology. Thus, the ABA biosynthesis pathway is also required for the full virulence of the obligate oomycete pathogen *H. arabidopsis* in terms of asexual spore production and proliferating hyphal growth, while ABA positively regulates resistance against necrotrophic *A. brassicicola* infection, indicating strikingly different roles of ABA in the interactions with bio/hemibiotrophs and necrotrophs. However, contradictory roles of ABA in other pathosystems have also been documented; for example, ABA suppresses resistance to necrotrophic pathogens, including *Botrytis cinerea*, *Fusarium oxysporum*, *Plectosphaerella cucumerina*, and *Erwinia chrysanthemi* (Audenaert et al., 2002; Anderson et al., 2004; Hernandez-Blanco et al., 2007; Asselbergh et al., 2008b), and ABA signaling may positively regulate the $RLM1_{Col}$ determined resistance to the hemibiotrophic fungal pathogen *Leptosphaeria maculans* and the *edr1*-mediated resistance to the obligate biotrophic powdery mildew pathogen *Golovinomyces cichoracearum* in Arabidopsis (Kaliff, et al., 2007; Wawrzynska et al., 2008). Hence, specific manifestations of the emerging new role for the abiotic stress signal ABA in modulating disease resistance may depend not only on pathogen lifestyle and overall infection biology but also on specialized features of each interaction, indicating that complex nuanced mechanisms underlie ABA modulation of plant biotic stresses.

ABA Modulation of Plant Disease Resistance Mechanisms

Plant receptors recognize extracellular MAMPs or intracellular virulence proteins derived from bacteria and activate multiple layers of defense to limit infection, whereas pathogenic bacteria are armed with a collection of effector molecules, including chemical and proteinacious factors to suppress plant defense responses and promote disease (Abramovitch et al., 2006). Recent work revealed that stomatal closure upon perception of MAMPs/bacteria is an important preinvasive innate immune response involving ABA signaling components to restrict bacterial entry (Melotto et al., 2006). However, at postinvasive stages,

ABA biosynthesis and signaling pathways may be targeted by *P. syringae* TTSS effectors to suppress the plant defense response (de Torres-Zabala et al., 2007). Our study on the *cds2-1D* mutant showed that activation of ABA biosynthesis weakened several plant defense systems against bacterial infection. The significantly enhanced growth of both virulent (*Pst* and *Psm*) and nonpathogenic (*hrcC*) *P. syringae* strains *on cds2-1D* in comparison to wild-type Col-0 indicates an ABA effect on suppression of the nonspecific basal resistance against bacterial infection, which is consistent with the observation that suppression of *Pst* growth by treatment with bacterial MAMP (flg22 peptide) was attenuated in the *cds2-D* mutant. Previous studies showed that treatment of Arabidopsis plants with flg22 peptide or the nonpathogenic *hrcC* strain of *Pst* may lead to callose-associated cell wall modification (Gómez-Gómez et al., 1999; Hauck et al., 2003), and this extracellular defense response is suppressed by wild-type pathogenic bacteria or overexpression in planta of bacterial TTSS effectors (Hauck et al., 2003; Kim et al., 2005). Recent data showed that wild-type *Pst* enhances callose deposition in Arabidopsis mutants impaired in ABA biosynthesis or signaling, and exogenous ABA suppresses flg22 peptide induced callose deposition in wild-type Arabidopsis seedlings (de Torres-Zabala et al., 2007, 2009; Clay et al., 2009), indicating a negative role of ABA in activation of callose deposition. However, early studies also showed that an ABA-dependent defense pathway mediates priming of callose deposition in β-amino-butyric acid (BABA)-induced resistance against necrotrophic pathogens (Ton and Mauch-Mani, 2004). Work is ongoing to understand whether callose deposition has a role in the observed disease phenotypes in the *cds2-1D* mutant. The enhanced growth of *P. syringae* strains carrying avirulence genes, including *avrRpm1*, indicated suppression of *R*-gene-dependent resistance expression in *cds2-1D* mutant plants. The systemic induction of *PR1* gene expression and acquired resistance to challenge by a normally virulent pathogen by local infection of avirulent *Pst* was significantly suppressed in *cds2-1D* mutant plants, indicating that ABA down-regulates systemic acquired resistance. Likewise, down-regulation of *RPS2/avrRpt2* determined resistance and chemically induced systemic acquired resistance were observed in ABA-treated or environmentally stressed plants (Mohr and Cahill, 2003; Yasuda et al., 2008). These findings highlight the importance of further investigation of the molecular events underlying ABA-modulated defense mechanisms.

ABA has a pivotal role in protection against water loss in plants under desiccation. In well-watered conditions, *cds2-1D* plants developed a phenotype of abnormal water soaking leaves in the dark, suggesting that ABA may potentially modulate physiological conditions in the leaves to facilitate bacterial growth. However, several lines of evidence argue against this hypothesis: first, the enhanced bacterial growth in *cds2-1D* plants differed considerably between wild-type *Pst*, *Psg*, and the mutant *hrcC* strains, indicating that the ABA effect on bacterial disease susceptibility is due to specific factor(s); second, the enhanced *Pst* growth was uncoupled from the abnormal water-soaking *cds2-1D* phenotype in detached leaves; third, in drought-stressed wild-type Col-0 plants, where ABA is reported to accumulate due to water deficiency, bacterial growth was increased compared to well-watered plants. Hence, ABA may influence plant resistance to bacterial infection by interacting with defense signaling networks or modulating effectors of defenses, rather than through a more general effect on physiological status. Interestingly, the enhanced growth of the TTSS$^-$ strain *hrcC* in *cds2-1D* was largely abolished when inoculated leaves were detached, thereby revealing an apparent association with the water-soaking phenotype. Previous studies showed that constitutive overexpression of an *NCED* gene in tomato (*Solanum lycopersicum*) plants leads to increased root exudation and flooding of leaf intercellular spaces with fluid containing much higher levels of ABA (Thompson et al., 2007). We speculate that a similar mechanism produced the water-soaking phenotype in *cds2-1D* plant and that the higher levels of apoplastic ABA led to the enhanced growth of *hrcC* bacteria, whereas detaching the leaf prevented the high levels of apoplastic ABA sustained by root exudation and abolished the enhanced growth of *hrcC* bacteria. This hypothesis is supported by the observation that feeding detached Col-0 leaves with ABA significantly enhanced growth of *hrcC* (Supplemental Fig. S1A) without the water-soaking symptom. Hence, in detached *cds2-1D* leaves, the enhanced growth differed between wild-type *Pst* and the *hrcC* mutant, implying that distinct mechanisms mediate ABA modulation of plant basal defenses against bacterial infection.

Possible Mechanisms of ABA Effect on Disease Resistance Signaling

SA and JA are well-established signal molecules mediating plant disease resistance, and our study showed a striking sequential induction of SA, ABA, and JA in *Pst*-inoculated Col-0 plants. Further investigation on the impact of endogenous ABA on JA and SA accumulation demonstrated a clear role of ABA in JA accumulation. Thus, compared to wild-type Col-0, JA accumulation is attenuated in ABA-deficient *aba3-1* mutant plants, whereas in *cds2-1D*, JA accumulation was substantially enhanced in response to bacterial infection. ABA is required for wound-induced JA accumulation in potato (*Solanum tuberosum*) and tomato plants (Penacortes et al., 1995), but the underlying mechanism is not well understood. Consistent with our findings, the attenuated JA accumulation was also observed in Arabidopsis *aba2* mutant plants in response to infection by necrotrophic pathogen *Pythium irregulare* (Adie et al., 2007), implying a general role of ABA in modulating biotic stress-induced JA accumulation. Our data also showed that the potentiated JA

induction in *cds2-1D* mutant plants was pathogenesis associated, and it is not yet clear whether this synergistic JA induction contributed to enhanced bacterial growth in *cds2-1D*, but it may help to explain the *A. brassicicola* disease phenotype of *aba3-1* and *cds2-1D* mutant plants, where plant resistance had been shown to be dependent on the JA signaling pathway (Thomma et al., 1998). Earlier reports indicated that ABA antagonizes JA/ET signaling to suppress defense against *F. oxysporum* in Arabidopsis (Anderson et al., 2004), whereas recent investigations showed that this ABA repression only affects a subset of JA/ET-regulated genes and that ABA predominately activates many ABA-specific and ABA/JA-related genes to promote plant defenses (Adie, et al., 2007). Nevertheless, ABA-dependent BABA-induced resistance to *A. brassicicola* infection does not require *COI1*, a key component of JA signaling, suggesting that additional signaling pathways mediate BABA-induced resistance to this pathogen (Ton and Mauch-Mani, 2004). Hence, further work is necessary to dissect the enhanced resistance to *A. brassicicola* in *cds2-1D* plants.

In contrast to its clear synergistic effect on JA induction, ABA showed complex antagonistic effects on SA induction, as both *aba3-1* and *cds2-1D* plants accumulated less SA in response to bacterial challenge. However, *PR1* expression is suppressed in SA-treated leaves or in systemic leaves of *cds2-1D* plants inoculated with avirulent bacteria, suggesting a damping effect on SA signaling. ABA has been shown to negatively regulate resistance and SA-dependent defense pathways in tomato-*Botrytis* interaction (Audenaert et al., 2002). Similarly, ABA treatment reduces the levels of conjugated SA induced by avirulent *Pseudomonas* and suppresses chemical-induced systemic acquired resistance in Arabidopsis plants (Mohr and Cahill, 2007; Yasuda et al., 2008), whereas in compatible interactions, bacterial effectors promote virulence by targeting ABA biosynthesis and signaling that rapidly antagonize SA-mediated defenses (de Torres-Zabala et al., 2007, 2009). Our data also showed that ABA levels in the *cds2-1D* mutant after bacterial challenge were rather constant and moderate in comparison with that of the wild-type Col-0 plant, implying that the timing of ABA accumulation is probably crucial to its impact on modulation of SA signaling, JA induction, and the final disease outcome.

The fact that *cds2-1D* with the phenotype of enhanced bacterial growth was isolated from the *NahG* background indicates that ABA may be involved in suppression of SA-independent mechanisms that regulate plant resistance to bacterial infection. This accords with the observation that flg22 peptide induced basal resistance, which is largely independent of SA, JA, or ET signaling pathways in suppressing growth of virulent bacteria (Zipfel et al., 2004; Tsuda et al., 2008), was also suppressed in the *cds2-1D* mutant. Likewise, the enhanced resistance to the soil-borne bacterium *Ralstonia solanacearum* in Arabidopsis mutants compromised in secondary cell wall formation is not dependent on SA, JA, or ET but closely associated with ABA biosynthesis and signaling pathways (Hernandez-Blanco et al., 2007). Recent findings that ABA acts as a proinflammatory cytokine in granulocytes (Bruzzone et al., 2007), which requires cADP-Rib as second messenger, is a reminiscent of some aspects of ABA signaling in the plant response to abiotic stresses (Wu et al., 1997; Sanchez et al., 2004). This highlights the possibility that ABA may play a conserved role in modulating immunity across the plant and animal kingdoms. Future studies on the molecular mechanisms of ABA modulation of pathogenesis may shed light on how plants integrate and fine-tune the complex responses to diverse biotic and abiotic stresses.

MATERIALS AND METHODS

Activation Tagging Screening

The activation tagging vector pJFAT260 was constructed on the backbone of a streamlined mini binary vector, pCB302 (Xiang et al., 1999), with a signature sequence of approximately 180 bp engineered between the tetramerized *CaMV* 35S enhancer and the T-DNA right border to facilitate subsequent identification of flanking DNA by thermal asymmetric interlaced PCR or IPCR. The *Agrobacterium tumefaciens* strain GV3101 (pMP90) was transformed with pJFAT260, and the resulting strain was used for floral dip transformation (Clough and Bent, 1998) of Arabidopsis (*Arabidopsis thaliana*) *NahG* plants (Gaffney et al., 1993). The activation-tagged transformants were selected by spraying T1 seedlings with 0.1% (v/v) of herbicide CHALLENGE 60 (containing 60 g/L of glufosinate ammonium). Three weeks later, the herbicide-resistant plants were hand-infiltrated with the *luxCDABE*-tagged *Psm*, and in planta bacterial growth was assayed as described (Fan et al., 2008). The T2 seeds of plants showing aberrant bacterial growth were kept for further characterization.

Plant Material and Pathogen Inoculations

All plants used for disease tests were grown at 23°C under short-day conditions (9 h of light and 15 h of dark). Bacterial strains used in this study were *Pseudomonas syringae* pv *maculicola* ES4326 (*Psm*), *Psm-avrRpm1*, *P. syringae* pv *tomato* DC3000 (*Pst*), *P. syringae* pv. *tomato* DC3000 *HrcC*$^-$ (*hrcC*), and *P. sryingae* pv *glycinea* (*Psg*). All strains were grown at 28°C on King's B plates containing appropriate antibiotics for selection. The resulting bacteria were collected and washed twice with water before inoculation of plants using a 1-mL syringe. The inoculated plants were kept in a growth room at 23°C, and bacterial growth was determined as described by Whalen et al. (1991).

The *Hyaloperonospora arabidopsis* Noco2 was maintained on Arabidopsis accession Col-0. Freshly harvested conidiospores were suspended in water (4×10^4 spores mL^{-1}) and sprayed onto 3-week-old plants. The inoculated plants were kept in a tray with a sealed lid to maintain high humidity in a short-day growth chamber at 19°C. Seven days after inoculation, leaves from four plants were pooled as one replicate and weighed in 15-mL tubes. Conidiospores were rinsed with 5 mL of distilled water by vortexing, and the spore numbers were determined using a hemocytometer. At least five replicates were analyzed in each assay.

The *Alternaria brassicicola* strain MUCL20297 was grown on potato dextrose agar for 10 d before harvest of conidiospores by washing the plate with distilled water. Fully expanded leaves of 5-week-old Arabidopsis plant were pierced with a pipet tip, and 5 μL of spore droplets (10^5 spores mL^{-1}) were applied on the wounds. The inoculated plants were kept at 20°C and 100% relative humidity for 7 d before photography.

Histochemical Staining

The *H. arabidopsis* inoculated leaves were stained with lactophenol trypan blue and destained with saturated chloral hydrate. The material was subsequently mounted on a slide in 60% glycerol and examined using a light microscope to monitor mycelium development (Koch and Slusarenko, 1990).

Gene Expression Studies

Total RNA was extracted from Arabidopsis leaf samples with TRIzol reagent (Invitrogen). Transcripts levels of *PR1* and *CDS2-1D* genes were determined by RNA gel blot analysis. Two micrograms of total RNA samples were resolved on formaldehyde-agarose gel and blotted onto nylon membrane and hybridized with ^{32}P-labeled probes prepared with the Random Primer Kit (Amersham). A template for the *PR1* probe was derived from an Arabidopsis cDNA clone (provided by K. Lawton, Syngenta), and *CDS2* template was amplified from Arabidopsis genomic DNA with oligos used for RT-PCR assay (see below). RNA loading was monitored by ethidium bromide staining or hybridization with ^{32}P-labeled ubiquitin gene probe. Expression of *NCED* genes was analyzed by RT-PCR with the following oligos: 5′-TCTTCTTACAATGCCGATGAGT-3′ and 5′-ACTCCGACGCCGTTTTGGTTG-3′ for *NCED2* (*At4g18350*) gene; 5′-CTCCAACGAAGATCAACAAGTCA-3′ and 5′-CACACGACCTGCTTCGCCAAA-3′ for *NCED3* (*At3g14440*) gene; 5′-GGAAATCCACACGCAGAACTA-3′ and 5′-TTGGTTTAAGCCTGGTTTAACAT-3′ for *NCED5* (*At1g30100*) gene; and 5′-CCTTCTGTCCCAAGATGCTCA-3′ and 5′-AGGTTATGCACGACAGGTTTC-3′ for *NCED9* (*At1g78390*) gene. Ten micrograms of total RNA was treated with the TURBO DNA-free kit (Ambion) to remove any contaminating genomic DNA, and 1 μg of treated RNA was reverse transcribed with SuperScript II reverse transcriptase (Invitrogen) in 20-μL reaction volume. Core mixtures that contain 1 μL of the resultant cDNA product were used to amplify individual *NCED* genes with PCR of 30 cycles. The ubiquitin gene was amplified with 25 cycles as reference to monitor cDNA input in each sample.

Construction of *NCED* Overexpression Transgenic Plants

The coding regions of Arabidopsis *NCED3* and *NCED5* gene were amplified with primers 5′-ATGGCTTCTTTCACGGCAACGGCT-3′/5′-ACTAGCAAACCGCACCCCAAAAG-3′ and 5′-ATACTCAAAATCTCTCGAGCTTC-3′/5′-ATTATGTGTCAACGTTTACTAGTT-3′, respectively, and cloned into pGEM T-easy vector for sequencing. The clones with correct sequence were used for subsequent cloning of the *NCED* genes into binary vector pCHF3 (Hajdukiewicz et al., 1994) for constitutive expression and pER8 (Zuo et al., 2000) for chemical-induced expression in Arabidopsis. The resultant constructs were used for floral dip transformation of Arabidopsis, and independent transgenic lines were selected by growing the T1 seeds on Murashige and Skoog plates containing appropriate antibiotics. Homozygous T2 lines with single T-DNA insertion indicated by segregation analysis were selected for further testing.

Extraction of Free SA, JA, and ABA

Arabidopsis leaves were snap-frozen with liquid nitrogen and lyophilized. About 10 mg of dried sample was ground to powder with liquid nitrogen and extracted at 4°C overnight with 4 mL of MeOH containing 40 ng of each d4-SA (catalog number D6322; CDN Isotopes) and d4-ABA (Plant Biotechnology Institute) and 100 ng dihydro JA (Tokyo Chemical Industry) as internal standards. The total extract was centrifuged (3,000*g*, 10 min), and the pellet was reextracted once with 2 mL of MeOH. The combined supernatant was dried with N_2 gas, and resultant residue was acidified with 2.5 mL of 5% (w/v) trichloroacetic acid and extracted twice with 5 mL of ethylacetate and cyclopentane mixture (1:1, v/v). The organic phase in each extraction was separated with aqueous phase by centrifugation (3,000*g*, 10 min) and combined to be dried with N_2 gas. The resulting residue was taken up in 500 μL of 20% MeOH for LC-MS analysis.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. ABA enhances disease susceptibility of Arabidopsis against *P. syringae* and *H. arabidopsis* infection.

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